MOLECULAR AND CELLULAR BIOLOGY, June 2008, p. 4080–4092 Vol. 28, No. 12
0270-7306/08/$08.00+0 doi:10.1128/MCB.02168-07


# DEC1 Modulates the Circadian Phase of Clock Gene Expression▽†

Ayumu Nakashima,[1,2] Takeshi Kawamoto,[1]* Kiyomasa K. Honda,[1,7] Taichi Ueshima,[1] Mitsuhide Noshiro,[1] Tomoyuki Iwata,[3,4] Katsumi Fujimoto,[1] Hiroshi Kubo,[4] Sato Honma,[5] Noriaki Yorioka,[6] Nobuoki Kohno,[2] and Yukio Kato[4,7]*

*Department of Dental and Medical Biochemistry,[1] Department of Molecular and Internal Medicine,[2] Department of Periodontal Medicine,[3] and Department of Advanced Nephrology,[6] Hiroshima University Graduate School of Biomedical Sciences, Hiroshima 734-8553, Japan; Natural Science Center for Basic Research and Development, Hiroshima University, Hiroshima 734-8551, Japan[4]; Department of Physiology, Hokkaido University Graduate School of Medicine, Sapporo 060-8638, Japan[5]; and Japan Science and Technology Agency, JST Innovation Plaza Hiroshima, Higashi-Hiroshima 739-0046, Japan[7]*

Received 5 December 2007/Returned for modification 1 January 2008/Accepted 5 April 2008

**DEC1 suppresses CLOCK/BMAL1-enhanced promoter activity, but its role in the circadian system of mammals remains unclear. Here we examined the effect of *Dec1* overexpression or deficiency on circadian gene expression triggered with 50% serum. Overexpression of *Dec1* delayed the phase of clock genes such as *Dec1*, *Dec2*, *Per1*, and *Dbp* that contain E boxes in their regulatory regions, whereas it had little effect on the circadian phase of *Per2* and *Cry1* carrying CACGTT E′ boxes. In contrast, *Dec1* deficiency advanced the phase of the E-box-containing clock genes but not that of the E′-box-containing clock genes. Accordingly, DEC1 showed strong binding and transrepression on the E box, but not on the E′ box, in chromatin immunoprecipitation, electrophoretic mobility shift, and luciferase reporter assays. *Dec1*$^{-/-}$ mice showed behavioral rhythms with slightly but significantly longer circadian periods under conditions of constant darkness and faster reentrainment to a 6-h phase-advanced shift of a light-dark cycle. Knockdown of *Dec2* with small interfering RNA advanced the phase of *Dec1* and *Dbp* expression, and double knockdown of *Dec1* and *Dec2* had much stronger effects on the expression of the E-box-containing clock genes. These findings suggest that DEC1, along with DEC2, plays a role in the finer regulation and robustness of the molecular clock.**

The mammalian molecular clock system consists of various clock genes and their protein products involved in interlocked feedback loops of transcriptional and translational regulation through clock elements such as CACGTG E-box, D-box, and ROR/REV-ERB binding elements (RORE) (18, 24). Among these regulatory sequences, the E box is thought to be the most important element in the molecular oscillatory system, since it is the binding site for the CLOCK/BMAL1 heterodimer, which up-regulates various clock genes, including *Dec1*, *Dec2*, *Per1*, *Dbp*, and *Rev-erb*α. In this regulatory system, PER, CRY, and DEC serve as negative factors for transcription from E-box-driven promoters, and the E-box-like element E′ box (CAC GTT) was recently shown to be involved in the direct regulation of *Per2* and *Cry1* genes by CLOCK/BMAL1 (1, 31, 34). RORE, on the other hand, is a clock element to which the transcriptional activators—RORα/RORβ/RORγ—and repressors—REV-ERBα/REV-ERBβ—bind, and ROR and REV-ERB regulate the circadian expression of *Bmal1*, *Clock*, *Npas2*, and *Cry1* via RORE (31).

In the mammalian clock system, DEC1 (also known as BHLHB2, STRA13, or SHARP2) and DEC2 (BHLHB3 or SHARP1) serve as transcriptional repressors for CLOCK/BMAL1-enhanced promoter activity, through binding to E boxes or interaction with BMAL1 (12, 14, 18, 26). Among suppressive factors for E boxes, DEC1 and DEC2 can bind directly to E boxes through their basic helix-loop-helix DNA binding domains (18, 26), although it remains unclear whether DEC1 and DEC2 also bind to the E′ boxes. In contrast, PER and CRY interact with the CLOCK/BMAL1 heterodimer but cannot bind directly to E/E′ boxes, since they have no DNA binding domain.

*Dec1* expression shows robust circadian rhythms in the suprachiasmatic nucleus and various peripheral tissues (7, 10, 14, 17, 18, 23), and it is further enhanced by light pulse, hypoxia, or growth factors, with the *Dec1* level gradually increasing during chondrogenic or osteogenic differentiation (6, 14, 15, 22, 25, 27–29). *Dec1* expression can also be elevated in various tissues by refeeding after starvation (17). The remarkable responsiveness of *Dec1* to these environmental factors raises the question of whether *Dec1* is involved in some type of cross-talk between environmental factors and the clock system, but the precise role of DEC1 in molecular clock systems remains unknown. Expression profiles of clock genes such as *Per2*, *Per3*, *Clock*, *Bmal1*, *Dbp*, and *Rev-erb*α did not show any appreciable changes in *Dec1*$^{-/-}$ mouse liver experiments, while 42 clock-controlled genes were affected in the *Dec1*$^{-/-}$ liver (11). On the other hand, Clockwork Orange (*cwo*), a *Drosophila* homolog of *Dec*, has been shown to be indispensable for the

* Corresponding author. Mailing address for Takeshi Kawamoto: Department of Dental and Medical Biochemistry, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima 734-8553, Japan. Phone and fax: 81-82-257-5629. E-mail: tkawamo@hiroshima-u.ac.jp. Mailing address for Yukio Kato: Natural Science Center for Basic Research and Development, Hiroshima University, Hiroshima 734-8551, Japan. Phone and fax: 81-82-257-5749. E-mail: ykato@hiroshima-u.ac.jp.

† Supplemental material for this article may be found at http://mcb.asm.org/.

▽ Published ahead of print on 14 April 2008.

maintenance of precise circadian rhythms of fly locomotor activity (16, 20, 21).

The object of this study was to determine precisely how DEC1 participates in circadian regulation in the presence of several other suppressive factors for the CLOCK/BMAL1 activity. Assessing the role of each individual clock gene in whole animals has proved difficult so far, since so many repressors are redundantly involved in the feedback inhibition of CLOCK/BMAL1 transactivation. In addition, metabolic status may change the expression profile of some clock genes (17). Considering the complexity of the clock system in vivo, mammalian cells in culture—showing circadian rhythms—may be a useful model for studies of DEC1. Previous studies have shown that brief treatment of various cells with 50% horse serum (or other compounds) triggers rhythmic expression of clock genes (2, 4, 5, 13, 32). In this report, we show that overexpression or deficiency of DEC1 in 50% serum-exposed cells selectively shifted the phase of several clock genes, including *Dec2*, *Per1*, *Dbp*, and *Rev-erbα*, by binding to the E boxes. And a deficiency of *Dec1* and *Dec2* markedly decreased the expression of several clock genes. In addition, locomotor activities of mice were affected by disruption of the *Dec1* gene. These findings suggest a selective and specific role for DEC1 and DEC2 in the precise adjustment and maintenance of the mammalian molecular clock system.

## MATERIALS AND METHODS

**Animals.** *Dec1*$^{-/-}$ mice were generated by inGenious Targeting Laboratory, Inc. (Stony Brook, NY). The 4.7-kb BamHI-BssSI genomic fragment of *Dec1*, which contains entire coding regions in exons 1 to 5, was replaced with a Neo cassette. The resulting chimeric mice were back-crossed to a C57BL/6J background for three generations. Male *Dec1*$^{-/-}$ mice ($n = 6$) and their wild-type littermates ($n = 6$) that were 4 to 6 months of age were individually housed under conditions of cycles of 12 h of light followed by 12 h of dark (LD) (lights on 8:00 to 20:00; light intensity in the light phase, 300 lx) for 2 or 3 weeks, after which the mice were released into constant darkness (DD) for 40 days. Locomotor activities were measured using infrared thermal sensors, and spontaneous locomotor activity data obtained were analyzed using Clock Lab software (Actimetrics Inc.). The free-running period of circadian behavioral rhythms was calculated by a chi-square periodogram using activity data from day 25 to 40 in DD. For phase-shifting experiments, the same mice which had been entrained to the LD cycle were subjected to a 6-h phase-advanced shift of an LD cycle. To determine the day when the reentrainment would be completed, phase-angle differences between light-off and activity-onset times of each day after the beginning of the phase shift were calculated for 20 days and compared with mean values of those for 5 days before the phase shift. Static significance was analyzed by a Bonferroni/Dunn post hoc test. All procedures were performed in compliance with the standard principles and guidelines for the care and use of laboratory animals of Hiroshima University Graduate School of Biomedical Sciences.

**Cell culture and serum shock.** Human fibroblast-like (stromal/mesenchymal stem) cells isolated from bone marrow were purchased from BioWhittaker (Walkersville, MD). Mouse embryonic fibroblasts were established from day 14.5 embryos of a *Dec1*$^{-/-}$ mouse and a wild-type littermate. Serum shocking of human fibroblast-like cells or mouse fibroblasts was performed as described previously (5). After a 2-h incubation with 50% horse serum, the medium was replaced with Dulbecco's modified Eagle's medium-high glucose (Gibco) supplemented with 5% fetal bovine serum. The cells were harvested at the indicated times and subjected to quantitative real-time reverse transcription-PCR (RT-PCR) analysis.

**Antibodies.** Rabbit antibodies against mouse DEC1 were produced by immunizing a synthetic peptide fragment of mDEC1:397–411 (Cys-Gln-Ala-Leu-Lys-Gln-Ile-Pro-Pro-Leu-Asn-Leu-Glu-Thr-Lys-Asp). The obtained antibodies were purified by affinity column chromatography. Anti-PER1 antibodies (KI044) were purchased from TransGenic Inc. Anti-CLOCK antibodies (catalog no. sc-6927) and control immunoglobulin G (normal rabbit immunoglobulin G) (catalog no. sc-2027) were from Santa Cruz Biotechnology.

**Adenovirus infection.** At 24 h after seeding the cells, infection with adenovirus expressing human *Dec1* (ad-Dec1) or adenovirus expressing *LacZ* (ad-LacZ) was performed as described previously (29). To control expression levels of overexpressed *Dec1* in both human fibroblast-like cells and mouse *Dec1*$^{-/-}$ fibroblasts, we examined the relationship between the mRNA level of *Dec1* and the multiplicities of infection (MOI) of the adenovirus. The mRNA level of overexpressed *Dec1* in the human cells was higher than that in the mouse cells at an MOI of 10 PFU/cell, but the levels in both cells were similar at an MOI of 50 and quite high (more than 10-fold higher) compared with the mRNA level in the noninfected human cells (see Fig. S1 in the supplemental material). Using this information, we infected human fibroblast-like cells and mouse *Dec1*$^{-/-}$ fibroblasts with ad-Dec1 or ad-LacZ at an MOI of 50. Adenovirus carrying the *LacZ* gene was generously supplied by Kohei Miyazono (The University of Tokyo) (9). At 6 h after the infection procedure, the culture medium was replaced with fresh medium and cultured for 3 days before being subjected to serum shock. The high level of expression of *Dec1* induced by serum shock was confirmed by quantitative RT-PCR analysis and Western blotting using anti-DEC1 antibodies.

**siRNA treatment.** Small interfering RNA (siRNA) directed against *Dec2* (5′-GACUAUUCCUCUUUGUAUAUG-3′ and 5′-UAUACAAAGAGGAAUAG UCCA-3′) and nonspecific control siRNA (Qiagen) were transfected into wild-type and *Dec1*$^{-/-}$ mouse fibroblasts by use of Lipofectamine 2000 (Invitrogen). The transfection efficiencies in these cells were more than 95% as determined with Alexa 488-labeled siRNA. Overall levels of suppression of *Dec2* mRNA in *Dec1*$^{-/-}$ cells and wild-type cells were 75.8% and 74.7%, respectively, as determined by averaging *Dec2* expression levels in those cells across the time points. At 72 h later, the transfected cells were subjected to serum shock for circadian rhythm detection.

**RNA extraction and real-time quantitative RT-PCR.** Total RNA was extracted from cultured cells by use of an RNeasy Mini kit (Qiagen) and was subjected to real-time quantitative RT-PCR analysis with an ABI Prism 7900 sequence detection system (Applied Biosystems), using TaqMan probes and primers as follows: 5′-6-carboxyfluorescein (FAM)–TATGGACACAGACAAAGATGAC CCTCATGG–6-carboxytetramethylrhodamine (TAMRA)-3′, 5′-GCTCCACTG ACTACCAAGAA-3′, and 5′-CTTCCCTTGCATTTTTTATCC-3′ for human *Bmal1*; 5′-FAM-AGATCAACTGCCTGGACAGCATCCTCAG-TAMRA-3′, 5′-AAGCCTCCAGCTGCTCCTACCA-3′, and 5′-GAGGTTGCAGCTCTCCA GGT-3′ for human *Per1*; 5′-FAM-TTTGAGGTGCTGATGGTGCGCTTTG-TAMRA-3′, 5′-CTTCCGTGACCTTTCTCAGCA-3′, and 5′-GGTGCGGCTT AGGAACATCAC-3′ for human *Rev-erbα*; and 5′-FAM-TGACAACAGACGG CAGCATCATCTATGTG-TAMRA-3′, 5′-GGCATTAGATGGCTTCGTCAT C-3′, and 5′-AAGGAGAGGTGTGATACTGTCGG-3′ for mouse *Npas2*. TaqMan probes and primers for human *Dec1*, *Dec2*, *Per2*, and *Dbp* and mouse *Dec1*, *Dec2*, *Bmal1*, *Clock*, *Dbp*, *Rev-erbα*, *Per1*, *Per2*, and *Cry1* were described previously (10, 15, 18, 23). TaqMan probes and primers for human *Cry1* and 18S RNA were obtained from Applied Biosystems. The values for mRNA levels, relative to internal control 18S RNA levels, represent the means ± standard error of the means (SEM) for three wells. Circadian rhythmicity was analyzed by one-way analysis of variance (ANOVA), and differences between the two groups were analyzed by two-way ANOVA. The peak times of mRNA levels in circadian rhythmicity were analyzed with cosinor fitting procedures, and the average period lengths from the first peak to the third peak were determined.

**ChIP assays.** Chromatin immunoprecipitation (ChIP) assays of *Dec1*, *Dec2*, *Per1*, *Dbp*, and *Rev-erbα* E boxes and the *Per2* E′ box were performed using a ChIP assay kit (Upstate) according to the manufacturer's instructions. DNA samples recovered from human fibroblast-like cells were subjected to PCR using gene-specific primers as follows: 5′-AGCTAGCAAGGGGATATTCC-3′ and 5′-TTGGAGCTACGTGTTCTACC-3′ were used for *Dec1*, 5′-GCCTTTGGG AAGATGAATAGC-3′ and 5′-AAGTGTGAAGCAGTTGGTCC-3′ for *Dec2*, 5′-AAAGAGGCGCAGCGTATCTC-3′ and 5′-TTGCATAATGCCGGGCAC TG-3′ for *Per1*, 5′-TTTGGGCACATTGAGGTCTC-3′ and 5′-CTGTACCTTT AAGCACCAGC-3′ for *Dbp*, 5′-CTCGTTACATAATGAGCTCC-3′ and 5′-CA GGAATGGCTCCATGTTAC-3′ for *Rev-erbα*, and 5′-ATGTAAAAAGAGCG ACGGGC-3′ and 5′-AGCAGCCCAAGGAACTTCC-3′ for *Per2*. Obtained PCR products were subjected to agarose gel electrophoresis. The experiments were repeated two to four times, and similar results were obtained each time.

**Plasmid constructions.** Oligonucleotides of three tandem sequences containing human *Per2* CACGTT E′ boxes (underlined) with 6 bp of flanking sequences (CGCGGTCACGTTTTCCAC) were subcloned into pGL3-TK (30). Construction of pGL3-TK containing three E boxes in the *Dec1* and *Per1* promoter and expression vectors for *Clock*, *Bmal1*, and *Dec1* has been described previously (14, 18). *Cry1* expression vector was generously supplied by Masaaki Ikeda, Saitama Medical School.

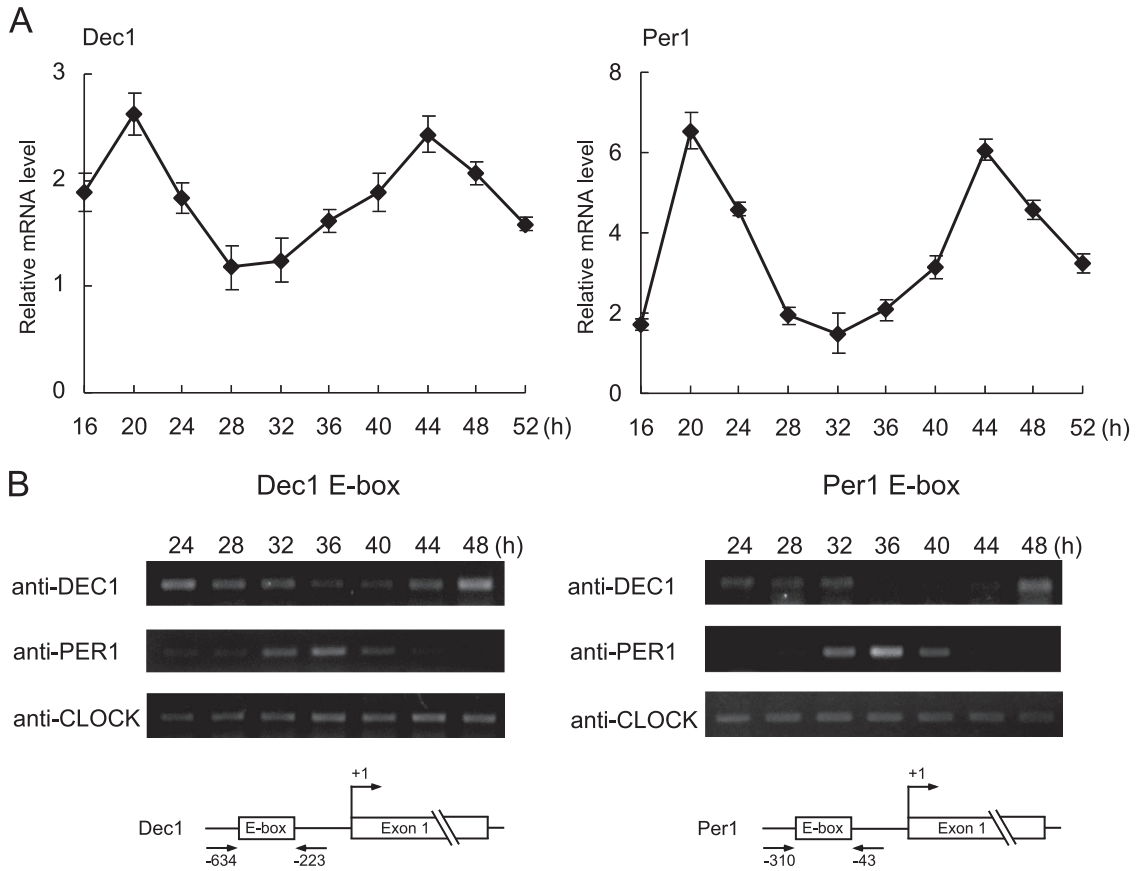



FIG. 1. Rhythmic expression of *Dec1* and *Per1* mRNA and binding of DEC1 and PER1 protein to CACGTG E-box in human fibroblast-like cells. (A) Rhythmic expression of clock genes (including *Dec1* and *Per1*) was induced by treatment with 50% horse serum for 2 h. Total RNA of the induced cells was extracted every 4 h and subjected to quantitative RT-PCR analysis of *Dec1* and *Per1* mRNA expression. (B) To observe the time course of DEC1, PER1, and CLOCK protein binding to E boxes in the *Dec1* and *Per1* promoters, the same cells were subjected to ChIP assays using anti-DEC1, anti-PER1, and anti-CLOCK antibodies. The extracted DNAs were amplified with primers specific for human *Dec1* or *Per1* and subjected to agarose gel electorphoresis. Locations of E boxes and PCR primers in the *Dec1* and *Per1* genes are shown. (C) ChIP assays of DEC1 binding to the E boxes of various clock genes in human fibroblast-like cells infected with *Dec1*-overexpressing or LacZ-overexpressing cells. After the serum shock, cells were harvested at the indicated times and subjected to ChIP assays using anti-DEC1 antibodies. The extracted DNAs were amplified with primers specific for human *Dec1*, *Dec2*, *Per1*, *Dbp*, and *Rev-erbα*. Locations of the E box and PCR primers in the *Dec2*, *Dbp*, and *Rev-erbα* genes are also shown. (D) Transcriptional induction of *Dec1* mRNA was examined at up to 12 h after the serum shock. (E) By use of Western blot analysis, expression levels of DEC1 protein in cells infected with ad-Dec1 or ad-LacZ were determined before and after the serum shock. (F) ChIP assays of CLOCK binding to the E box and E′ box in *Dec1*-overexpressing cells and control LacZ-expressing cells. The samples precipitated with anti-CLOCK antibodies were amplified with primers specific for human *Dec1* and *Per2*. Locations of the E′ box and PCR primers in the *Per2* gene are also shown. IgG, immunoglobulin G.

**Transient transfection and luciferase reporter assay.** NIH 3T3 cells were seeded at $3 \times 10^4$ cells per 16-mm-diameter well 24 h before transfection. Luciferase reporter plasmid (2 ng per well) and mouse *Clock* expression and *Bmal1* expression vectors (50 ng each per well) were cotransfected with or without *Dec1* or *Cry1* expression vectors (5 or 10 ng per well) by use of Lipofectamine 2000. As an internal standard, 0.05 ng of pRL-SV40 (Promega) was cotransfected. The total concentration of DNA was adjusted to 112.05 ng per well with an empty vector (pcDNA3.1/Zeo; Promega). After incubation for 4 h, the medium was replaced with fresh medium. The cells were incubated for a further 48 h and subjected to a luciferase reporter assay using a dual-luciferase reporter assay system (Promega). Luciferase activities were normalized by internal control activities. The values represent the means ± SEM of the results obained for four wells. Statistical significance was analyzed by a Student *t* test.

**Electrophoretic mobility shift assays (EMSA).** Human DEC1 protein was synthesized using TNT-coupled reticulocyte lysate systems (Promega). The double-stranded oligonucleotides of the DEC1 E box (5′-agCGTTGTCCAACACGTGAGACTCATctca-3′) and the Per2 E′ box (5′-CGCGCGCGGTCACGTTTTCCACTATGctca-3′) were labeled using [$^{32}$P]dCTP (GE Healthcare) and a DNA polymerase I Klenow fragment (New England Biolabs). Synthesized human DEC1 protein was incubated with approximately 40 fmol (20,000 cpm) of $^{32}$P-labeled probe for 10 min at room temperature in 15 μl of 10 mM Tris-HCl (pH 8.0)–0.5 mM dithiothreitol–10% glycerol–1 μg of poly(dI-dC)–50 mM NaCl–5 mM $MgCl_2$, and then the mixtures were subjected to 5% polyacrylamide gel electrophoresis in 12 mM Tris-HCl–125 mM glycine–1 mM EDTA electrophoresis buffer. In competition experiments, a 25-, 50-, or 100-fold molar excess of unlabeled *Dec1* E box or *Per2* E′ box was added.

## RESULTS

**Rhythmic expression of *Dec1* and its profile of binding to CACGTG E box in living cells.** Since brief treatment of various cells with 50% horse serum triggers rhythmic expression of clock genes in culture (5), we used this technique to determine whether an expression profile of *Dec1* mRNA would have circadian rhythmicity in human fibroblast-like cells (bone marrow stromal cells). In these cells, the *Dec1* mRNA levels were

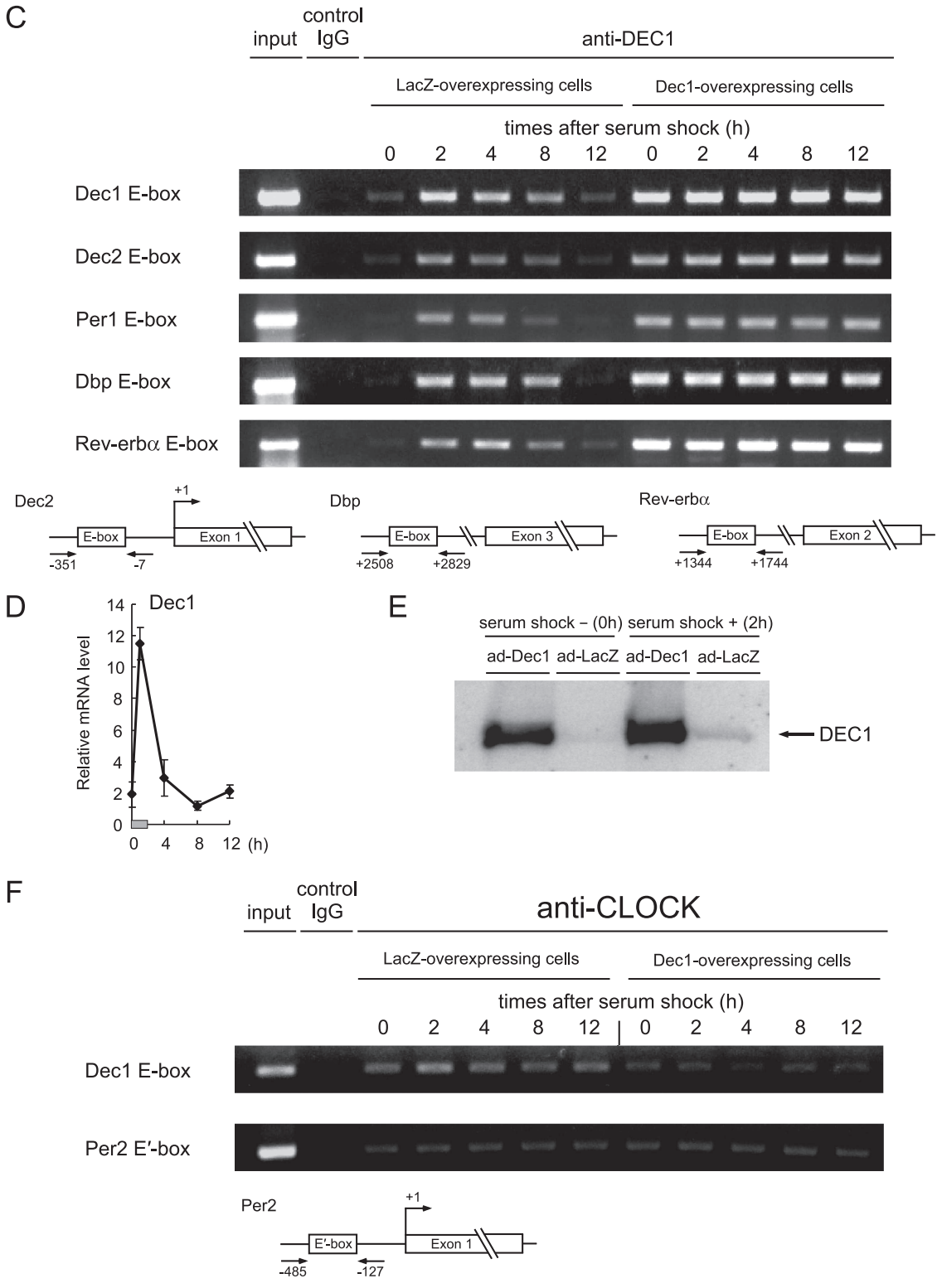



FIG. 1—*Continued.*

higher than those in human skin fibroblasts (data not shown). The expression pattern of *Dec1* after 50% serum treatment for 2 h showed a robust circadian rhythm, with peaks at approximately 20 h and 44 h after the start of serum stimulation (Fig. 1A). The circadian mRNA profile was similar to that of *Per1*, with the peak observed at the same phase.

Although DEC1 is a transcriptional repressor that binds to the E boxes of some clock genes in EMSA (12, 14, 18), the

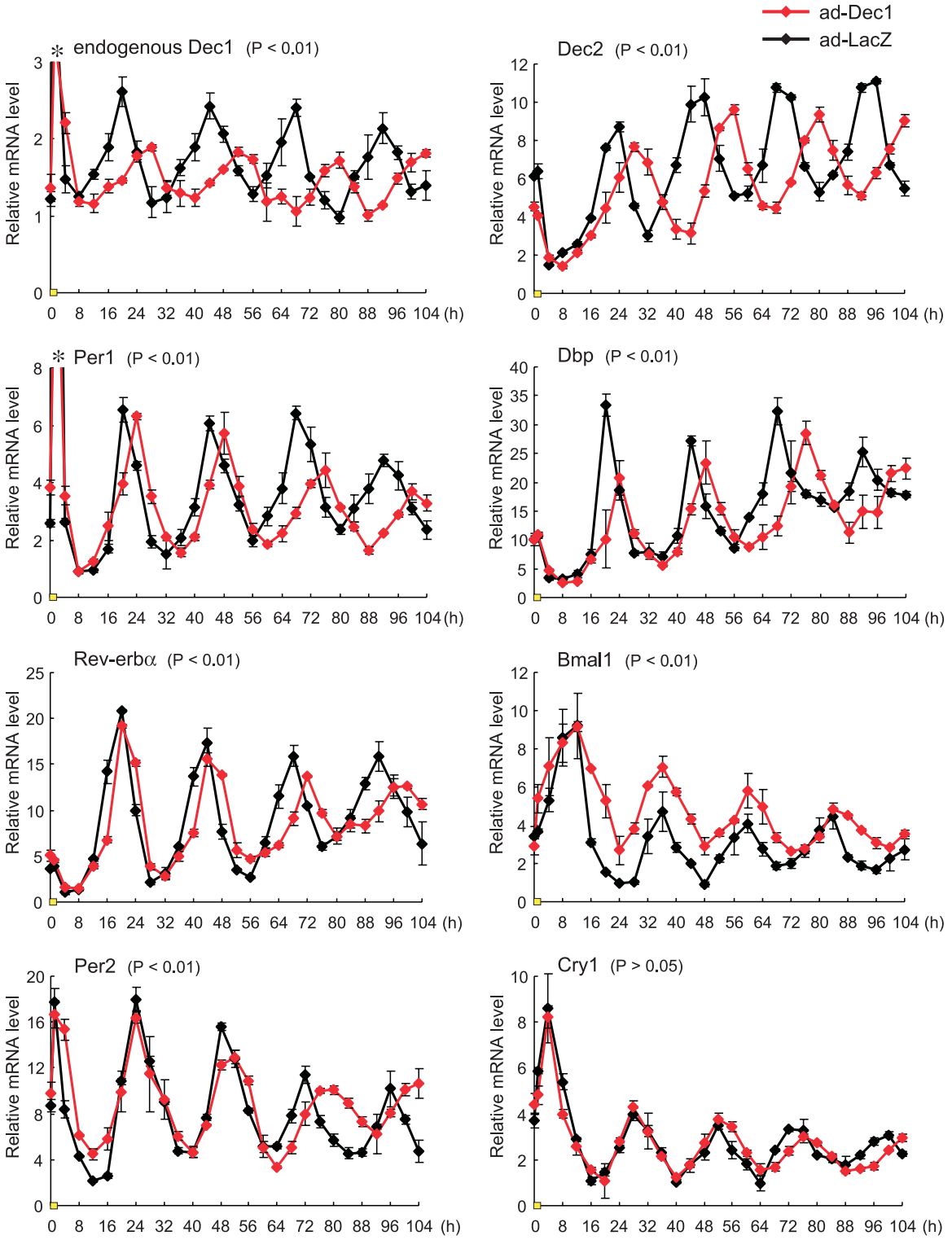



FIG. 2. The phase delays of circadian expression of E-box-containing clock genes caused by *Dec1* overexpression after serum shock. Human fibroblast-like cells were infected with ad-Dec1 (red lines) or ad-LacZ (black lines). Three days later, the cells were incubated with 50% horse serum for 2 h, and total RNA was extracted from these cells at the indicated times after the start of serum shock and subjected to quantitative RT-PCR analysis. Expression profiles of all examined clock genes showed significant circadian rhythms (one-way ANOVA; $P < 0.01$). Immediate-early induction of *Dec1* and *Per1* expression is indicated by asterisks. Numbers in parentheses represent differences between the two groups (two-way ANOVA; $P < 0.01$, $P < 0.05$, or $P > 0.05$).

TABLE 1. Effect of overexpression (ad-Dec1) and deficiency (*Dec1*$^{-/-}$) of *Dec1* and knockdown (siRNA) of *Dec2* on circadian expression of clock genes at the first peak after serum shock[a]

| Gene expressed | % Phase difference change (h) and up- or down-regulation result for indicated treatment and cell category | | | | | Presence or absence of conserved E box (CACGTG) |
|---|---|---|---|---|---|---|
| | ad-Dec1 | | Dec1 KO (Dec1$^{+/+}$ [mouse]) | Dec2 siRNA | | |
| | Dec1$^{+/+}$ (human) | Dec1$^{-/-}$ (mouse) | | Dec1$^{+/+}$ (mouse) | Dec1$^{-/-}$ (mouse) | |
| *Dec1* | +6.3, ↓↓ | ND | ND | −3.9, ↑ | ND | Present |
| *Dec2* | +5.6, ↓ | +3.6, ↓ | −2.8, ↑ | ND | ND | Present |
| *Per1* | +2.3 | +2.9 | −2.0 | n.s. | −3.3, ↓ | Present |
| *Dbp* | +3.3, ↓ | +1.9 | −2.5 | −1.0 | −1.3, ↓↓ | Present |
| *Rev-erbα* | +2.3 | +1.8 | −2.6 | −1.5 | −2.0, ↓ | Present |
| *Per2* | n.s. | n.s. | n.s. | n.s. | −1.1 | Absent |
| *Cry1* | n.s. | n.s. | n.s. | n.s. | n.s. | Absent |

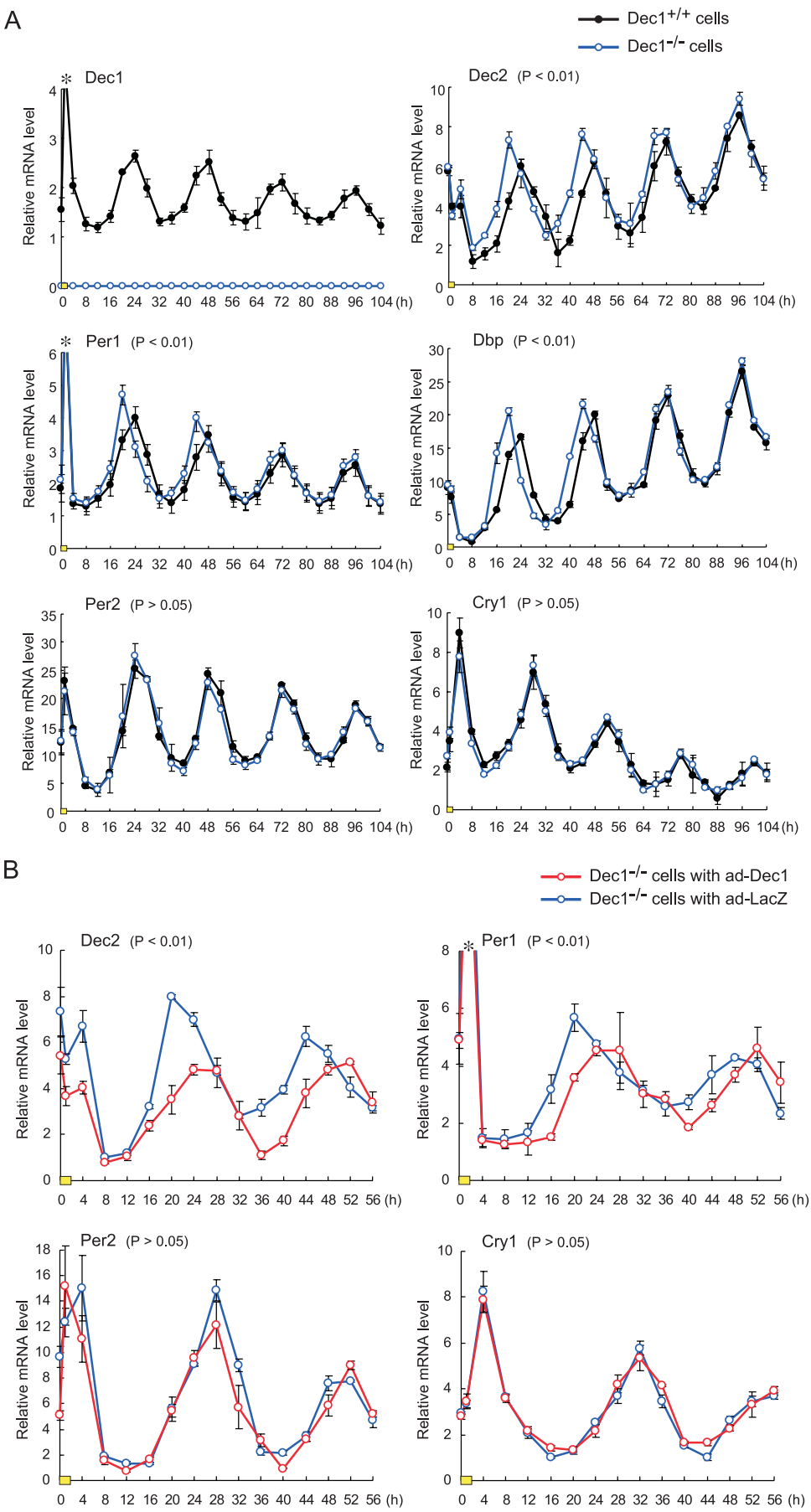
[a] Human fibroblast-like cells and mouse wild-type (*Dec1*$^{+/+}$) or *Dec1*$^{-/-}$ fibroblasts were infected with ad-Dec1 or transfected with *Dec2* siRNA. Differences in clock gene expression profiles for the two groups in the period from 8 h to 36 h after serum shock were analyzed by two-way ANOVA. Values represent the phase difference (in hours) between the gene expression rhythms of the experimental and control groups at the first peak. Positive and negative values indicate advanced and delayed phase differences from the circadian peak of each control gene, respectively. KO, knockout; n.s., no significant difference; ↓, lesser down-regulation (20 to 40% decrease of area under the curve) of average expression level; ↓↓, greater down-regulation (>40%) of average expression level; ↑, up-regulation (20 to 40%) of average expression level; ND, not determined.

status of its binding to the E boxes in living cells had not yet been demonstrated. Therefore, we examined the profile of DEC1 binding to the CACGTG E boxes in *Dec1* and *Per1* promoters by use of ChIP assays with anti-DEC1 antibodies. The results revealed that DEC1 bound to the E boxes in the fibroblast-like cells and that the DEC1 binding profile exhibited circadian rhythmicity. The binding levels of DEC1 protein were maximal when the mRNA levels of *Dec1* and *Per1* began to decrease (at 24 and 48 h after serum shock) (Fig. 1B). In contrast, binding of PER1 protein to the same E boxes was maximal when the expression levels of *Dec1* and *Per1* began to increase (at 36 h after serum shock). These findings demonstrated that DEC1 and PER1 bind to the E boxes in almost opposite phases of the circadian rhythms. On the other hand, profiles of binding of CLOCK to the E boxes in the *Dec1* and *Per1* promoters did not show significant circadian rhythmicity. The absence of circadian rhythmicity in CLOCK binding to E boxes in human fibroblasts corresponds to the previous observation in mice liver by Lee et al. (19).

**Binding of DEC1 to E boxes in the various clock genes in *Dec1*-overexpressing and control cells.** DEC1 binding to E boxes of various clock genes in human cells infected with *ad-Dec1* or control *LacZ* was also examined. The DEC1 binding to E boxes of *Dec1*, *Dec2*, *Per1*, *Dbp*, and *Rev-erbα* was observed by ChIP analysis using anti-DEC1 antibodies and specific primers for each gene. The DEC1 binding to the E boxes in the control *LacZ*-expressing cells markedly increased within 2 h after serum shock and started to decrease at 4 h (Fig. 1C). The level of DEC1 protein that bound to the E boxes declined to a minimal level at 12 h, while the DEC1 protein binding level in *Dec1*-overexpressing cells maintained high levels for at least 12 h, irrespective of the presence or absence of serum stimulation. The profile of DEC1 binding to the E boxes in *LacZ*-expressing cells correlated with the changes in the expression level of *Dec1* mRNA after serum stimulation (Fig. 1D), and the enhanced binding of DEC1 protein to the E boxes in *Dec1*-overexpressing cells coincided with constitutively increased *Dec1* protein levels in the cells (Fig. 1E). To investigate the effect of overexpressed DEC1 on the binding of CLOCK/BMAL1 heterodimer to the E box, we performed ChIP assays using anti-CLOCK antibodies. DEC1 overexpression suppressed—but did not abolish—the binding of CLOCK to the E box in the *Dec1* promoter (Fig. 1F). However, it had little effect on CLOCK binding to the *Per2* E′ box.

**Effect of *Dec1* overexpression on circadian rhythms of clock gene expression.** Human fibroblast-like cells overexpressing Dec1 (ad-Dec1) or control *LacZ* (ad-LacZ) were exposed to 50% serum for 2 h, after which total RNA was extracted every 4 h. Expression profiles of clock genes—including *Dec1*, *Dec2*, *Per1*, *Dbp*, *Rev-erbα*, *Bmal1*, *Per2*, and *Cry1*—showed robust circadian rhythmicity in both *Dec1*-overexpressing and *LacZ*-expressing cells (one-way ANOVA; $P < 0.01$) (Fig. 2). However, *Dec1* overexpression delayed the phases of circadian profiles of endogenous *Dec1* and *Dec2* mRNA for 6.3 and 5.6 h, respectively, relative to those seen with control cells, with first peak around 24 h after serum shock (Fig. 2 and Table 1), and the period lengths of *Dec1* and *Dec2* in *Dec1*-overexpressing cells (25.8 and 25.6 h) were about 2 h longer than those seen with control cells (23.9 and 23.8 h). The overexpression also delayed the peak phases of *Per1*, *Dbp*, and *Rev-erbα*—which carry E boxes in their regulatory regions—for 2.3, 3.3, and 2.3 h, respectively, at the first peak, and the period lengths of *Per1*, *Dbp*, and *Rev-erbα* were 25.4, 25.5, and 25.8 h, respectively, for the *Dec1*-overexpressing cells and 23.8, 23.7, and 24.0 h, respectively, for the control cells. Furthermore, overexpression of *Dec1* reduced the expression levels of *Dec1*, *Dec2*, and *Dbp*, probably because of the continuous interaction of DEC1 with E boxes. In contrast, overexpression of *Dec1* had little effect on the initial phases of circadian expression of *Per2*, *Cry1*, and *Bmal1*, which do not contain conserved E boxes in their regulatory regions. However, the period lengths of *Per2* and *Cry1* expression were also lengthened by *Dec1* overexpression. The lengths were 25.5 and 24.0 h, respectively, for the *Dec1*-overexpressing cells and 23.9 and 23.0 h, respectively, for the control cells, resulting in an apparent phase delay at the third or fourth peak. In addition, *Dec1* overexpression increased the expression level of *Bmal1* mRNA.

**The phase advance of E-box-containing clock gene expression rhythms in *Dec1*$^{-/-}$ cells.** If the phase delay of the clock gene expression caused by *Dec1* overexpression is indeed related to the physiological role of DEC1, DEC1 deprivation should have the opposite effect on the phase of clock gene


A
Dec1+/+ cells
Dec1-/- cells
Dec1
Dec2 (P < 0.01)
Per1 (P < 0.01)
Dbp (P < 0.01)
Per2 (P > 0.05)
Cry1 (P > 0.05)
Relative mRNA level
0 8 16 24 32 40 48 56 64 72 80 88 96 104 (h)
B
Dec1-/- cells with ad-Dec1
Dec1-/- cells with ad-LacZ
Dec2 (P < 0.01)
Per1 (P < 0.01)
Per2 (P > 0.05)
Cry1 (P > 0.05)
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 (h)

expression. To test this hypothesis, we isolated fibroblasts from embryos of *Dec1*$^{-/-}$ and wild-type mice. *Dec1* expression in the *Dec1*$^{-/-}$ fibroblasts was abolished, whereas it showed a robust circadian rhythmicity in wild-type fibroblasts after serum shock (Fig. 3A). The expression profiles of *Dec2*, *Per1*, and *Dbp* were significantly different in the *Dec1*$^{-/-}$ fibroblasts, with the first peaks detected 2.8, 2.0, and 2.5 h, respectively, earlier than those of wild-type (*Dec1*$^{+/+}$) cells. Interestingly, long-term observation revealed that the phase advance in the circadian expression rhythms of *Dec2*, *Per1*, and *Dbp* was gradually reduced each circadian cycle, and finally no differences were detected at the fourth cycle. Accordingly, the period lengths of *Dec2*, *Per1*, and *Dbp* circadian expression in the *Dec1*$^{-/-}$ cells were longer than in the wild-type cells. The lengths of *Dec2*, *Per1*, and *Dbp* were 24.7, 25.2, and 25.0 h, respectively, in the *Dec1*$^{-/-}$ cells and 23.6, 24.4, and 24.0 h, respectively, in the wild-type cells. In contrast, *Dec1* deficiency had little effect on the phases and periods of *Per2* and *Cry1* circadian expression.

In the next experiment, we looked at whether the phase advance observed in *Dec1*$^{-/-}$ cells could be restored by *Dec1* overexpression. Overexpression of human *Dec1* in mouse *Dec1*$^{-/-}$ cells phase-delayed the expression rhythms of *Dec2*, *Per1*, and *Dbp* for 3.6, 2.9, and 1.9 h, respectively, at the first peak and significantly reduced the expression level of *Dec2* (Fig. 3B and Table 1). In contrast, the phases of *Per2* and *Cry1* expression rhythms were not altered. These findings proved that DEC1 is indeed involved in control of the circadian phase and amplitude of the E-box-containing clock genes, with weaker effects on those of E′-box-containing clock genes.

**Effects of *Dec2* knockdown in *Dec1*$^{-/-}$ cells on circadian expression of clock genes.** To determine whether DEC2 has similar effects on the expression of clock genes, siRNA was used to knock down *Dec2* expression. Treatment with siRNA against *Dec2* resulted in a phase advance of circadian expression rhythm of *Dec1* and *Dbp* for 3.9 and 1.0 h, respectively, at the first peak and slightly increased the expression level of *Dec1* (Fig. 4A and Table 1), although it did not change the expression of *Per1* and the second peak time of *Dbp*. In addition, transfection of *Dec2* siRNA into *Dec1*$^{-/-}$ cells markedly decreased the amplitude of *Dbp* and *Per1* circadian expression and further advanced their phases by 1.3 and 3.3 h, respectively (Fig. 4B and Table 1), whereas it had only weak effects on the phases and expression levels of *Per2* and *Cry1* (Table 1). The disruption of both *Dec1* and *Dec2* expression had a much greater effect on circadian expression of *Dbp* and *Per1* than that of either *Dec1* or *Dec2* alone, and knockdown of both *Dec1* and *Dec2* decreased the expression levels of *Rev-erbα*, *Bmal1*, *Clock*, and *Npas2* (Fig. 4C and Table 1).

**Stronger direct action of DEC1 on E box than on E′ box.** Although the circadian expression of the *Per2* gene is regulated by CLOCK/BMAL1, the gene does not contain the CACGTG E box in its regulatory region. The elements responsible for the regulation are CACGTT E′ boxes. Since the effect of *Dec1* overexpression or deficiency on the circadian expression of *Per2* was much less than that on CACGTG E-box-carrying clock genes, we compared the effects of DEC1 on transcriptional activities from E-box- and E′-box-carrying promoters. In luciferase reporter assays, DEC1 abolished the CLOCK/BMAL1-enhanced transcriptional activities of *Dec1* and *Per1* E boxes, whereas it had far less effect on the promoter activity of the *Per2* E′-box-containing construct (Fig. 5A). In contrast, CRY1 abolished the CLOCK/BMAL1-enhanced transcriptional activity of both the *Per2* E′ box and the *Dec1* and *Per1* E boxes. Accordingly, in ChIP analysis the *Per2* E′-box region did not coprecipitate with anti-DEC1 antibodies in either the presence or the absence of the stimulation by 50% serum, but the E-box regions in *Dec1* and *Per1* promoters did coprecipitate with anti-DEC1 antibodies (Fig. 5B). On the other hand, both the *Per2* E′ box and the E boxes of *Dec1* and *Per1* coprecipitated with anti-PER1 antibodies 2 h after serum stimulation. In EMSA, DEC1 bound to both the E box and the E′ box, but the intensity of the shifted band of the E box associated with DEC1 protein was much greater than that of the E′ box (Fig. 5C). In addition, unlabeled *Dec1* E-box oligonucleotides competed with a $^{32}$P-labeled *Dec1* E box or *Per2* E′ box more effectively than unlabeled *Per2* E′-box oligonucleotides did. These findings indicate that the binding affinity of DEC1 for the *Dec1* E box is higher than that for the *Per2* E′ box. The stronger binding of DEC1 to E boxes probably selectively delayed the circadian phases of clock genes containing E boxes, whereas PER1 indirectly bound to both the E box and the E′ box via protein-protein interaction with CLOCK/BMAL1 after post-translational modification.

**Effect of *Dec1* deficiency on circadian behavioral rhythms of mice.** To clarify the role of DEC1 in vivo, we compared the circadian phenotype of *Dec1*$^{-/-}$ mice with that of wild-type mice by monitoring their spontaneous locomotor activities under DD as well as LD conditions. *Dec1*$^{-/-}$ mice showed robust activity rhythms in LD, with phases of activity onset and end not significantly different from those of wild-type mice (data not shown), but the average circadian period in DD was 24.10 ± 0.05 h (mean ± SEM; $n = 6$), which was significantly longer than that of wild-type mice (23.95 ± 0.03 h; $n = 6$ [$P < 0.05$; Student $t$ test]) (Fig. 6A and B). We also subjected *Dec1*$^{-/-}$ and wild-type mice to a simulated jet lag experiment by phase-shifting an LD cycle. Following a 6-h phase-advancing shift of the LD cycle, behavioral rhythms of *Dec1*$^{-/-}$ mice showed gradual phase advance and were reentrained on day 7 after the phase shift, while it took 10 days for the wild-type mice to reach complete reentrainment after the 6-h phase advance of the LD cycle (Fig. 6C and D; also see Table S1 in the supplemental material). The activity onset times of *Dec1*$^{-/-}$ mice from day 3

FIG. 3. Effect of *Dec1* deficiency on E-box-containing clock genes and effects of *Dec1* overexpression in *Dec1*$^{-/-}$ cells. (A) *Dec1*$^{-/-}$ (blue lines) and wild-type (*Dec1*$^{+/+}$; black lines) fibroblasts obtained from a *Dec1*$^{-/-}$ mouse and a wild-type littermate were treated with 50% horse serum. The mRNA expression levels (means ± SEM; $n = 3$) of *Dec1*, *Dec2*, *Per1*, *Dbp*, *Per2*, and *Cry1* were determined every 4 h by quantitative RT-PCR analysis. Immediate-early induction of *Dec1* and *Per1* expression is indicated by asterisks. (B) *Dec1*$^{-/-}$ fibroblasts were infected with ad-Dec1 (red lines) or ad-LacZ (blue lines) and subjected to serum shock for circadian expression of clock genes. The mRNA levels were determined. Numbers in parentheses represent differences between the two groups (two-way ANOVA; $P < 0.01$, $P < 0.05$, or $P > 0.05$).

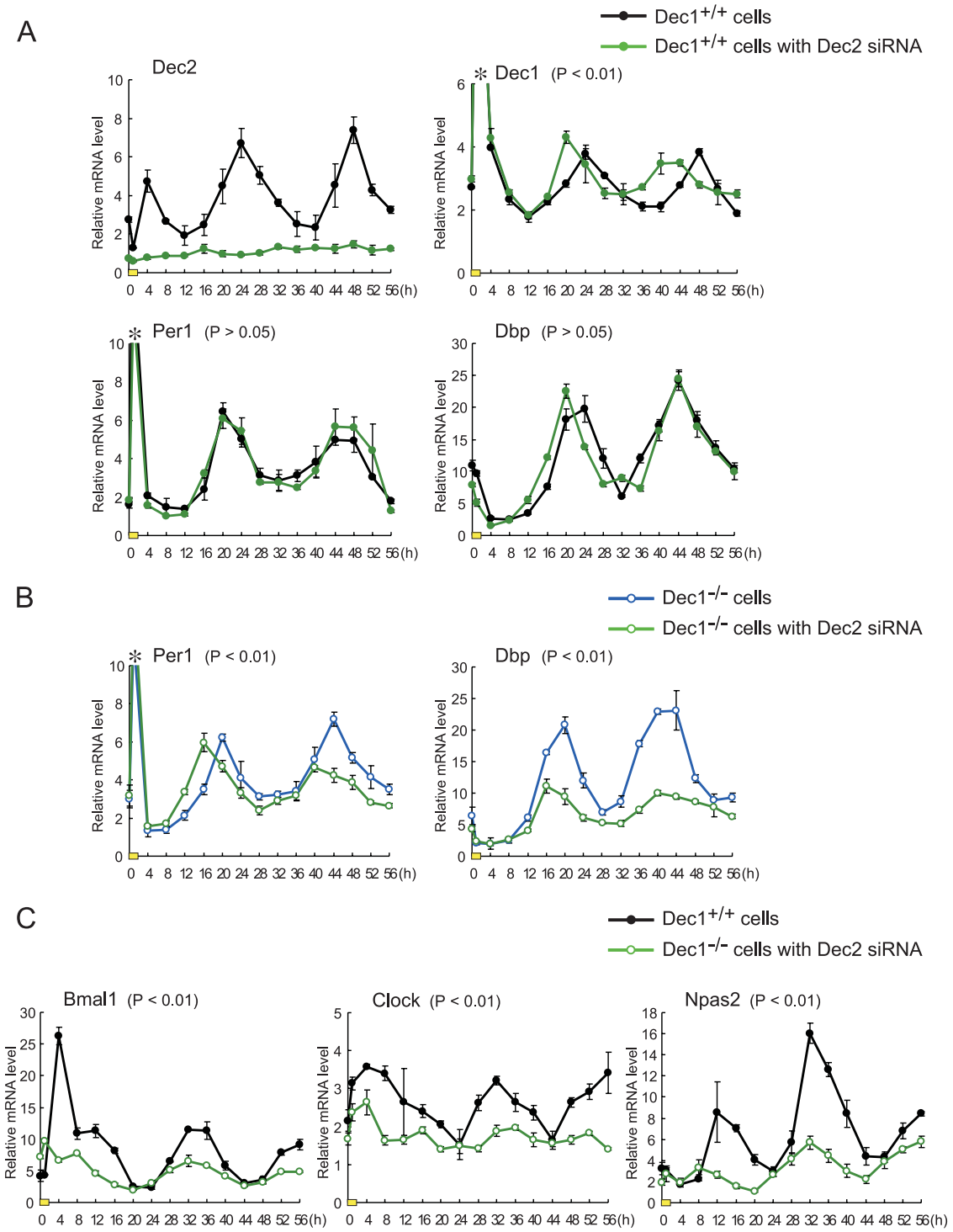



FIG. 4. Effect of *Dec2* knockdown on circadian expression of clock genes. (A) *Dec2* (green lines) or nonspecific control (black lines) siRNA was transfected into wild-type (*Dec1*$^{+/+}$) fibroblasts. At 72 h later, the cells were treated with 50% horse serum. The mRNA expression levels (means ± SEM; $n = 3$) of *Dec2*, *Dec1*, *Per1*, and *Dbp* were determined by quantitative RT-PCR analysis. Immediate-early induction of *Dec1* and *Per1* expression is indicated by asterisks. (B) Expression levels (means ± SEM; $n = 3$) of *Per1* and *Dbp* in *Dec1*$^{-/-}$ fibroblasts transfected with *Dec2* (green lines) or control (blue lines) siRNA were determined. (C) Expression levels (means ± SEM; $n = 3$) of *Bmal1*, *Clock*, and *Npas2* in *Dec1*$^{-/-}$ fibroblasts transfected with *Dec2* siRNA (green lines) were compared with those in wild-type (*Dec1*$^{+/+}$) cells transfected with control siRNA (black lines). Numbers in parentheses represent differences between the two groups (two-way ANOVA; $P < 0.01$, $P < 0.05$, or $P > 0.05$).

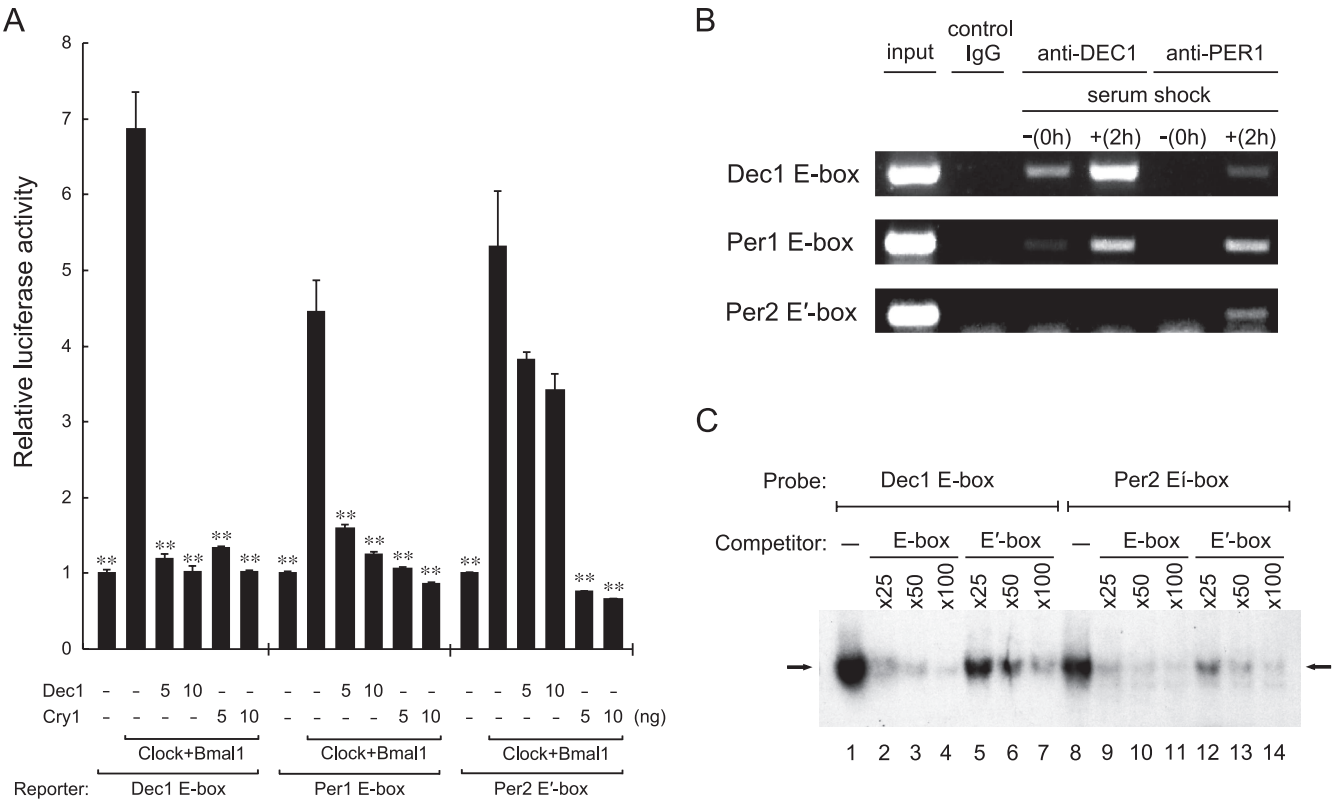



FIG. 5. Comparison of clock gene regulation profiles of E-box-containing clock genes such as *Dec1* and *Per1* genes to that of the E′-box-containing *Per2* gene. *P* values were calculated by using Student *t* tests (**, $P < 0.01$). (A) Luciferase reporter assays of reporter genes containing three tandem copies of the *Dec1* or *Per1* E box or the *Per2* E′ box were performed as described in Materials and Methods. The reporter constructs were cotransfected with *Clock* and *Bmal1* expression vectors (50 ng each) together with various amounts of *Dec1* or *Cry1* expression vectors as indicated. Luciferase activities (means ± SEM; $n = 4$) were measured after a 48-h incubation. (B) ChIP assays of human fibroblast-like cells by use of anti-DEC1 and anti-PER1 antibodies were performed before, and 2 h after, serum shock. (C) Binding of DEC1 to the *Dec1* E box (CACGTG) and the *Per2* E′ box (CACGTT) was examined by EMSA. The $^{32}$P-labeled *Dec1* E-box (lanes 1 to 7) or *Per2* E′-box (lanes 8 to 14) probe was incubated with human DEC1 protein synthesized by an in vitro transcription/translation system. Competition experiments were performed using a 25-, 50-, or 100-fold molar excess of unlabeled *Dec1* E box (lanes 2 to 4 and 9 to 11) or *Per2* E′ box (lanes 5 to 7 and 12 to 14). Arrows indicate the shifted bands of radiolabeled *Dec1* E box or *Per2* E′ box bound to DEC1 protein. IgG, immunoglobulin G.

to day 9 after the phase shift were significantly earlier than those of wild-type mice (Fig. 6D). Thus, the reentrainment of behavioral rhythms in *Dec1*$^{-/-}$ mice was significantly faster than that in wild-type mice.

## DISCUSSION

In the present study, we demonstrated that *Dec1* overexpression in both human and mouse cells caused a phase delay in circadian rhythms of the expression of CACGTG E-box-containing genes such as *Dec1*, *Dec2*, *Per1*, *Dbp*, and *Rev-erbα* (Fig. 2 and 3B and Table 1) in the first cycle after serum shock but that *Dec1* deficiency advanced the circadian phase of E-box-containing genes (Fig. 3A and Table 1). In addition to these phenomena, *Dec1* overexpression decreased the expression level or amplitude of *Dec1*, *Dec2*, and *Dbp*, whereas *Dec1* deficiency increased the expression of *Dec2* mRNA. Thus, overexpression and deficiency of *Dec1* had virtually opposite effects on the phase and expression levels of clock genes. On the other hand, *Dec1* overexpression had less effect on the *Per2* and *Cry1* circadian expression driven by CACGTT E′ boxes (Fig. 2 and 3B and Table 1) than that on the E-box-containing genes. Supporting this observation, luciferase reporter assays showed a lower inhibitory effect of DEC1 on the *Per2* promoter, and ChIP and EMSA analyses confirmed the weaker binding of DEC1 to E′ box of *Per2* (Fig. 5). DEC1 binding to E′ box in living cells may be too weak to form a stable complex in ChIP analysis or to cause the phase shift of *Per2* or *Cry1* expression in the initial peak. Furthermore, *Dec1* overexpression suppressed CLOCK binding to the E box but did not affect CLOCK binding to the E′ box (Fig. 1F). Thus, circadian rhythms of E-box-containing and E′-box-containing genes were dissociated within the same cells by overexpression or suppression of *Dec1*, although the dissociated phases in *Dec1*$^{-/-}$ cells may resynchronize after three or more circadian cycles.

The direct binding affinity of DEC1/DEC2, but not PER/CRY, for E-box sequences is the most intriguing difference between DEC and PER/CRY as negative components of the molecular clock. We found here that DEC1 action is selective for the clock genes containing E boxes, while PER/CRY can suppress the expression of both E-box- and E′-box-containing clock genes. *Dec1* overexpression thus resulted in phase shifts of all of E-box-containing genes examined in this study,

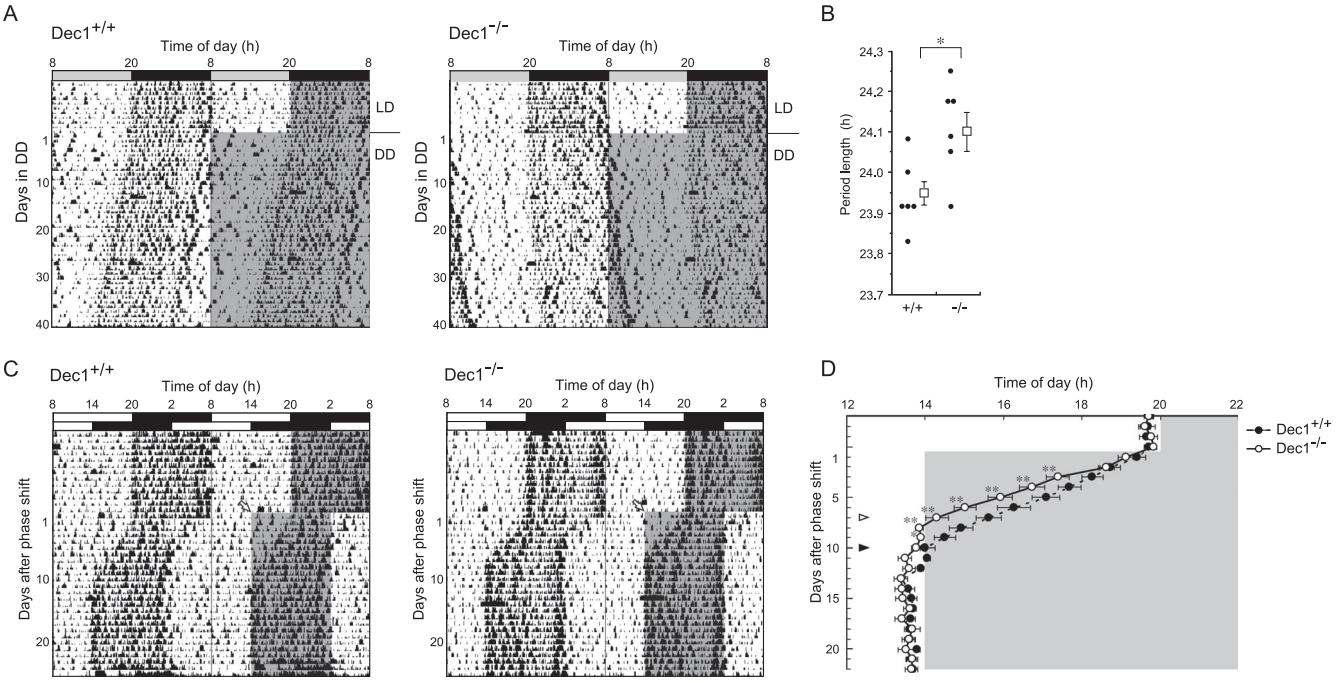



FIG. 6. Circadian phenotypes of *Dec1*$^{-/-}$ mice. (A) Representative double-plotted locomotor activity of a wild-type mouse (*Dec1*$^{+/+}$) and a *Dec1*$^{-/-}$ mouse. Mice were maintained in LD for the first 12 days of the record and then released into DD. A gray background in the right half of the depiction of activity data indicates the dark periods. Black and gray horizontal bars at the top of each activity record indicate the dark and light phases in LD. (B) Distribution of the free-running periods of wild-type (+/+) and *Dec1*$^{-/-}$ mice. Closed circles indicate individual periods, and open boxes indicate the average period (± SEM) for a genotype. *P* values were calculated by using Student *t* tests (∗, $P < 0.05$). (C) Effects of a 6-h phase advance on activity onset. Representative double-plotted activity data for a wild-type mouse (*Dec1*$^{+/+}$) and a *Dec1*$^{-/-}$ mouse before and after the phase-advance shift of an LD cycle by 6 h. Open arrows indicate the beginning of the phase advance. Black and white horizontal bars at the top of each panel depicting activity data indicate dark and light phases of an LD cycle, respectively, before and after the phase shift. (D) Phase shifts of activity onset times before and after a 6-h phase advance of an LD cycle. The onset times from day 1 to day 22 were significantly different between *Dec1*$^{-/-}$ and wild-type mice (two-way ANOVA; $P < 0.01$); the onset phases were significantly earlier from day 3 to day 9 for *Dec1*$^{-/-}$ mice compared with wild-type control results (∗, $P < 0.05$; ∗∗, $< 0.01$ [Bonferroni/Dunn post hoc test]). Open and closed arrowheads indicate the days when reentrainment was completed (day 7 for *Dec1*$^{-/-}$ mice; day 10 for wild-type mice). The activity onset time under LD entrainment was slightly earlier (by 0.2 ± 0.1 h) than light-off time for *Dec1*$^{-/-}$ mice, a result which was not significantly different from that seen with wild-type mice (0.2 ± 0.2 h).

whereas forced expression of *Per2* at a high level almost abolished the circadian rhythms of endogenous *Per2* and *Dbp* expression in NIH 3T3 cells and, at a relatively low level, reduced the amplitude of the gene expression but had little effect on the circadian phase (33). Among a number of suppressive factors—DEC1, DEC2, PER1, PER2, PER3, CRY1, and CRY2—some may have redundant functions, and some distinct ones, in the molecular clock system. These findings indicate that DEC1/DEC2 and PER/CRY have distinct roles derived from discrete selectivity characteristics with respect to the E boxes and E′ boxes.

*Dec1* overexpression markedly enhanced DEC1 binding to the E boxes of various clock genes (Fig. 1C), whereas these clock genes in *Dec1*-overexpressed cells still displayed rhythmic expression. In agreement with these observations, *Dec1* overexpression suppressed—but did not abolish—CLOCK binding to the E box (Fig. 1F). A CLOCK/BMAL1 heterodimer and DEC1 may compete for the E box, depending upon the equilibrium between these transcription factors. Even in the absence of appropriate rhythms of DEC1 action, the PER2 and CRY1 loops (Fig. 7) seem virtually intact, a state which could be sufficient to maintain rhythmicity.

DEC1 may modulate the circadian phases of some clock genes without influence on amplitude of the rhythmicity. *Dec1* expression is known to be induced by various exogenous or endogenous stimuli—including light pulses, hypoxia, growth factors, and feeding. And since different tissues respond to different environmental signals for their tissue-specific functions, DEC1 may constitute a distinct input pathway through E-box-containing clock genes such as *Dec1*, *Dec2*, *Per1*, and *Dbp* for modulation of circadian clocks in tissue-specific manners.

In the present study, free-running periods of behavioral rhythms of *Dec1*$^{-/-}$ mice in DD were slightly but significantly longer than those of wild-type mice (Fig. 6A and B). In addition, *Dec1*$^{-/-}$ mice reentrained to a 6-h phase advance of an LD cycle faster than wild-type mice did (Fig. 6C and D). Accordingly, knockdown of Clockwork Orange, a *Drosophila* homolog of *Dec*, showed circadian behavioral rhythms with 2- to 3-h-longer periodicities than the wild type (16, 20, 21); *Npas2*$^{-/-}$ or *Ror*$\alpha^{+/-}$ mice also showed faster reentrainment following a 4-h advance of an LD cycle (3, 8), possibly because of weakened clock controls. Both *Dec* and Clockwork Orange are therefore necessary for precise control of circadian rhythms, although the effect of *Dec1* deficiency on the behavioral rhythms of mice seems to be less robust than that of

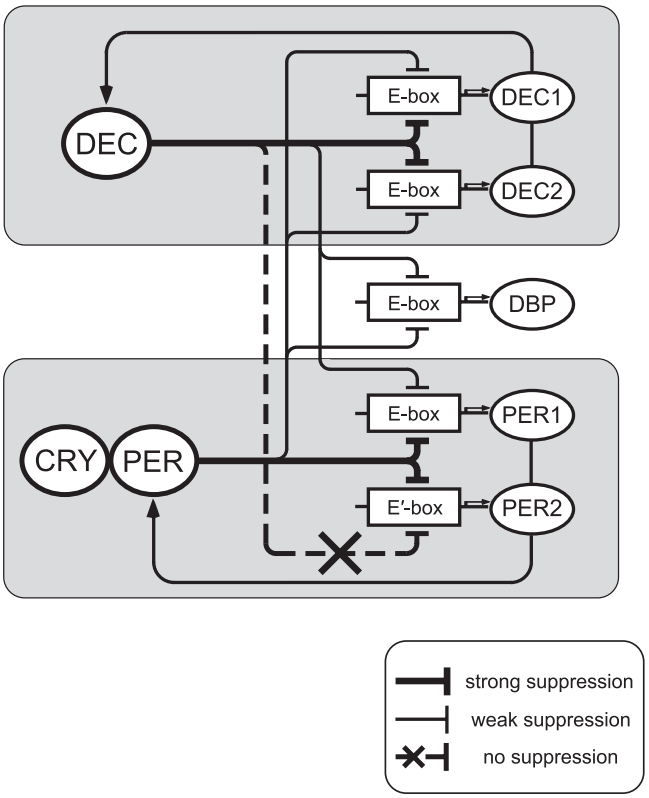



FIG. 7. A model of DEC and PER functions. DEC1 directly binds to the CACGTG E boxes of clock genes and causes a phase delay in their circadian rhythms, whereas, because of very weak binding to the CACGTT E′ box, it does not change the circadian phase of *Per2* expression. The effects of DEC on *Dec1* and *Dec2* expression (DEC loop) are much stronger than the effects on *Per1* and *Dbp* expression, while the suppressive effect of PER on *Per1* and *Per2* expression (PER/CRY loop) seems to be stronger than that on *Dec1* and *Dec2* expression.

Clockwork Orange for *Drosophila* species (16, 20, 21). However, this difference may be derived from overlapping functions of *Dec1* and *Dec2* in maintaining and entraining circadian rhythms. *Drosophila* species seem to have only one Clockwork Orange.

The variation of the circadian phases caused by *Dec1* overexpression among E-box-containing clock genes was unexpected. *Dec1* overexpression had its greatest effect on the phases of *Dec1* and *Dec2*, a moderate effect on those of *Per1* and *Dbp*, and the least effect on that of *Rev-erbα* (Fig. 2 and Table 1). Thus, DEC1 appears to be more effective in regulating its own feedback loop (DEC loop) than in regulating the interlocked loop with PER and CRY (Fig. 7). Similarly, PER and CRY may be more effective in their own feedback loop (PER/CRY loop) than in the interlocked loops. *Per2* overexpression had a greater suppressive effect on endogenous *Per2* expression than on *Dbp* expression (33). The varying effectiveness of DEC1 with respect to *Dec1*/*Dec2*, *Per1*/*Dbp*, and *Rev-erbα* expression in living cells may be accounted for by the presence of different numbers of E boxes at different positions, different binding affinities of DEC1 for the E boxes with different surrounding sequences, and/or different numbers of other clock elements.

Gene expression patterns of *Dec1* and *Per1* after serum shock were remarkably similar (Fig. 2), but we found that the patterns of timing of DEC1 and PER1 binding to E boxes were different (Fig. 1B). DEC1 may rapidly translocate into the nucleus and bind to E boxes after its transcriptional induction, whereas there is a long time lag before the occurrence of PER1 binding to E boxes via protein-protein interaction with a CLOCK/BMAL1 heterodimer after elevated transcription of *Per1*.

Unexpectedly, *Bmal1*, *Clock*, and *Npas2* expression was down-regulated by the knockdown of both *Dec1* and *Dec2* (Fig. 4C and Table 1). The mechanism underlying the decreases in *Bmal1*, *Clock*, and *Npas2* levels in the *Dec1* and *Dec2* doubly deficient cells is not yet known, but *Ror* mRNA levels were also reduced in the cells (data not shown). Since RORs are transcriptional activators for *Bmal1*, *Clock*, and *Npas2*, reduction of *Ror* expression is assumed to induce down-regulation of *Bmal1*, *Clock*, and *Npas2*.

In conclusion, our findings indicate a physiological role for DEC1 in the circadian rhythms of clock gene expression in living cells. DEC1 altered the circadian phase of various clock genes through its direct interaction with E boxes. The degree of the phase delay induced by *Dec1* overexpression was most prominently displayed in results associated with the circadian expression of *Dec2*, and forced expression of *Dec1* had little effect on the expression of E′-box-containing genes, suggesting that the DEC loop is partially separated from the PER/CRY loop. We also found that DEC2 had similar effects on E-box-containing clock genes and that the double knockdown of *Dec1* and *Dec2* markedly decreased the amplitude of the circadian expression of several clock genes, emphasizing the essential role of the DEC loop in the molecular clock system.

## ACKNOWLEDGMENTS

The work was supported by grants-in-aid for science from the Ministry of Education, Culture, Sport, Science and Technology of Japan. K.K.H. and T.U. were Iwadare Scholarship Fellows.

## REFERENCES

1. **Akashi, M., T. Ichise, T. Mamine, and T. Takumi.** 2006. Molecular mechanism of cell-autonomous circadian gene expression of Period2, a crucial regulator of the mammalian circadian clock. Mol. Biol. Cell **17:**555–565.
2. **Akashi, M., and E. Nishida.** 2000. Involvement of the MAP kinase cascade in resetting of the mammalian circadian clock. Genes Dev. **14:**645–649.
3. **Akashi, M., and T. Takumi.** 2005. The orphan nuclear receptor RORα regulates circadian transcription of the mammalian core-clock *Bmal1*. Nat. Struct. Mol. Biol. **12:**441–448.
4. **Balsalobre, A., S. A. Brown, L. Marcacci, F. Tronche, C. Kellendonk, H. M. Reichardt, G. Schutz, and U. Schibler.** 2000. Resetting of circadian time in peripheral tissues by glucocorticoid signaling. Science **289:**2344–2347.
5. **Balsalobre, A., F. Damiola, and U. Schibler.** 1998. A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell **93:**929–937.
6. **Boudjelal, M., R. Taneja, S. Matsubara, P. Bouillet, P. Dolle, and P. Chambon.** 1997. Overexpression of Stra13, a novel retinoic acid-inducible gene of the basic helix-loop-helix family, inhibits mesodermal and promotes neuronal differentiation of P19 cells. Genes Dev. **11:**2052–2065.
7. **Butler, M. P., S. Honma, T. Fukumoto, T. Kawamoto, K. Fujimoto, M. Noshiro, Y. Kato, and K. Honma.** 2004. Dec1 and Dec2 expression is disrupted in the suprachiasmatic nuclei of Clock mutant mice. J. Biol. Rhythms **19:**126–134.
8. **Dudley, C. A., C. Erbel-Sieler, S. J. Estill, M. Reick, P. Franken, S. Pitts, and S. L. McKnight.** 2003. Altered patterns of sleep and behavioral adaptability in NPAS2-deficient mice. Science **301:**379–383.
9. **Fujii, M., K. Takeda, T. Imamura, H. Aoki, T. K. Sampath, S. Enomoto, M. Kawabata, M. Kato, H. Ichijo, and K. Miyazono.** 1999. Roles of bone morphogenetic protein type I receptors and Smad proteins in osteoblast and chondroblast differentiation. Mol. Biol. Cell **10:**3801–3813.
10. **Furukawa, M., T. Kawamoto, M. Noshiro, K. K. Honda, M. Sakai, K.**

**Fujimoto, S. Honma, K. Honma, T. Hamada, and Y. Kato.** 2005. Clock gene expression in the submandibular glands. J. Dent. Res. **84:**1193–1197.
11. **Grechez-Cassiau, A., S. Panda, S. Lacoche, M. Teboul, S. Azmi, V. Laudet, J. B. Hogenesch, R. Taneja, and F. Delaunay.** 2004. The transcriptional repressor STRA13 regulates a subset of peripheral circadian outputs. J. Biol. Chem. **279:**1141–1150.
12. **Hamaguchi, H., K. Fujimoto, T. Kawamoto, M. Noshiro, K. Maemura, N. Takeda, R. Nagai, M. Furukawa, S. Honma, K. Honma, H. Kurihara, and Y. Kato.** 2004. Expression of the gene for Dec2, a basic helix-loop-helix transcription factor, is regulated by a molecular clock system. Biochem. J. **382:** 43–50.
13. **Hirota, T., T. Okano, K. Kokame, H. Shirotani-Ikejima, T. Miyata, and Y. Fukada.** 2002. Glucose down-regulates Per1 and Per2 mRNA levels and induces circadian gene expression in cultured Rat-1 fibroblasts. J. Biol. Chem. **277:**44244–44251.
14. **Honma, S., T. Kawamoto, Y. Takagi, K. Fujimoto, F. Sato, M. Noshiro, Y. Kato, and K. Honma.** 2002. Dec1 and Dec2 are regulators of the mammalian molecular clock. Nature **419:**841–844.
15. **Iwata, T., T. Kawamoto, E. Sasabe, K. Miyazaki, K. Fujimoto, M. Noshiro, H. Kurihara, and Y. Kato.** 2006. Effects of overexpression of basic helix-loop-helix transcription factor Dec1 on osteogenic and adipogenic differentiation of mesenchymal stem cells. Eur. J. Cell Biol. **85:**423–431.
16. **Kadener, S., D. Stoleru, M. McDonald, P. Nawathean, and M. Rosbash.** 2007. Clockwork Orange is a transcriptional repressor and a new Drosophila circadian pacemaker component. Genes Dev. **21:**1675–1686.
17. **Kawamoto, T., M. Noshiro, M. Furukawa, K. K. Honda, A. Nakashima, T. Ueshima, E. Usui, Y. Katsura, K. Fujimoto, S. Honma, K. Honma, T. Hamada, and Y. Kato.** 2006. Effects of fasting and re-feeding on the expression of dec1, per1, and other clock-related genes. J. Biochem. (Tokyo) **140:**401–408.
18. **Kawamoto, T., M. Noshiro, F. Sato, K. Maemura, N. Takeda, R. Nagai, T. Iwata, K. Fujimoto, M. Furukawa, K. Miyazaki, S. Honma, K. Honma, and Y. Kato.** 2004. A novel autofeedback loop of Dec1 transcription involved in circadian rhythm regulation. Biochem. Biophys. Res. Commun. **313:**117–124.
19. **Lee, C., J. P. Etchegaray, F. R. Cagampang, A. S. Loudon, and S. M. Reppert.** 2001. Posttranslational mechanisms regulate the mammalian circadian clock. Cell **107:**855–867.
20. **Lim, C., B. Y. Chung, J. L. Pitman, J. J. McGill, S. Pradhan, J. Lee, K. P. Keegan, J. Choe, and R. Allada.** 2007. *clockwork orange* encodes a transcriptional repressor important for circadian-clock amplitude in *Drosophila*. Curr. Biol. **17:**1082–1089.
21. **Matsumoto, A., M. Ukai-Tadenuma, R. G. Yamada, J. Houl, K. D. Uno, T. Kasukawa, B. Dauwalder, T. Q. Itoh, K. Takahashi, R. Ueda, P. E. Hardin, T. Tanimura, and H. R. Ueda.** 2007. A functional genomics strategy reveals clockwork orange as a transcriptional regulator in the Drosophila circadian clock. Genes Dev. **21:**1687–1700.
22. **Miyazaki, K., T. Kawamoto, K. Tanimoto, M. Nishiyama, H. Honda, and Y. Kato.** 2002. Identification of functional hypoxia response elements in the promoter region of the DEC1 and DEC2 genes. J. Biol. Chem. **277:**47014–47021.
23. **Noshiro, M., T. Kawamoto, M. Furukawa, K. Fujimoto, Y. Yoshida, E. Sasabe, S. Tsutsumi, T. Hamada, S. Honma, K. Honma, and Y. Kato.** 2004. Rhythmic expression of DEC1 and DEC2 in peripheral tissues: DEC2 is a potent suppressor for hepatic cytochrome P450s opposing DBP. Genes Cells **9:**317–329.
24. **Roenneberg, T., and M. Merrow.** 2003. The network of time: understanding the molecular circadian system. Curr. Biol. **13:**R198–R207.
25. **Rossner, M. J., J. Dorr, P. Gass, M. H. Schwab, and K. A. Nave.** 1997. SHARPs: mammalian enhancer-of-split- and hairy-related proteins coupled to neuronal stimulation. Mol. Cell. Neurosci. **9:**460–475.
26. **Sato, F., T. Kawamoto, K. Fujimoto, M. Noshiro, K. K. Honda, S. Honma, K. Honma, and Y. Kato.** 2004. Functional analysis of the basic helix-loop-helix transcription factor DEC1 in circadian regulation. Interaction with BMAL1. Eur. J. Biochem. **271:**4409–4419.
27. **Shen, M., T. Kawamoto, M. Teramoto, S. Makihira, K. Fujimoto, W. Yan, M. Noshiro, and Y. Kato.** 2001. Induction of basic helix-loop-helix protein DEC1 (BHLHB2)/Stra13/Sharp2 in response to the cyclic adenosine monophosphate pathway. Eur. J. Cell Biol. **80:**329–334.
28. **Shen, M., T. Kawamoto, W. Yan, K. Nakamasu, M. Tamagami, Y. Koyano, M. Noshiro, and Y. Kato.** 1997. Molecular characterization of the novel basic helix-loop-helix protein DEC1 expressed in differentiated human embryo chondrocytes. Biochem. Biophys. Res. Commun. **236:**294–298.
29. **Shen, M., E. Yoshida, W. Yan, T. Kawamoto, K. Suardita, Y. Koyano, K. Fujimoto, M. Noshiro, and Y. Kato.** 2002. Basic helix-loop-helix protein DEC1 promotes chondrocyte differentiation at the early and terminal stages. J. Biol. Chem. **277:**50112–50120.
30. **Sueyoshi, T., T. Kawamoto, I. Zelko, P. Honkakoski, and M. Negishi.** 1999. The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene. J. Biol. Chem. **274:**6043–6046.
31. **Ueda, H. R., S. Hayashi, W. Chen, M. Sano, M. Machida, Y. Shigeyoshi, M. Iino, and S. Hashimoto.** 2005. System-level identification of transcriptional circuits underlying mammalian circadian clocks. Nat. Genet. **37:**187–192.
32. **Yagita, K., and H. Okamura.** 2000. Forskolin induces circadian gene expression of rPer1, rPer2 and dbp in mammalian rat-1 fibroblasts. FEBS Lett. **465:**79–82.
33. **Yamamoto, Y., K. Yagita, and H. Okamura.** 2005. Role of cyclic mPer2 expression in the mammalian cellular clock. Mol. Cell. Biol. **25:**1912–1921.
34. **Yoo, S. H., C. H. Ko, P. L. Lowrey, E. D. Buhr, E. J. Song, S. Chang, O. J. Yoo, S. Yamazaki, C. Lee, and J. S. Takahashi.** 2005. A noncanonical E-box enhancer drives mouse Period2 circadian oscillations in vivo. Proc. Natl. Acad. Sci. USA **102:**2608–2613.